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## **Targeting of sodium-glucose cotransporters with phlorizin inhibits polycystic kidney disease progression in Han:SPRD rats**

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**Abstract:** Renal tubular epithelial cell proliferation and transepithelial cyst fluid secretion are key features in the progression of polycystic kidney disease (PKD). As the role of the apical renal sodium-glucose cotransporters in these processes is not known, we tested whether phlorizin inhibits cyst growth and delays renal disease progression in a rat model of PKD. Glycosuria was induced by subcutaneous injection of phlorizin in male heterozygous (Cy/+) and wild-type Han:SPRD rats. Phlorizin induced immediate and sustained glycosuria and osmotic diuresis in these rats. Cy/+ rats treated with phlorizin for 5 weeks showed a significant increase in creatinine clearance, a lower 2-kidneys/body weight ratio, a lower renal cyst index, and reduced urinary albumin excretion as compared with vehicle-treated Cy/+ rats. Measurement of Ki67 staining found significantly lower cell proliferation in dilated tubules and cysts of Cy/+ rats treated with phlorizin, as well as a marked inhibition of the activated MAP kinase pathway. In contrast, the mTOR pathway remained unaltered. Phlorizin dose dependently inhibited MAP kinase in cultured tubular epithelial cells from Cy/+ rats. Thus, long-term treatment with phlorizin significantly inhibits cystic disease progression in a rat model of PKD. Hence, induction of glycosuria and osmotic diuresis (glycuresis) by renal sodium-glucose cotransporters inhibition could have a therapeutic effect in polycystic kidney disease. *Kidney International* advance online publication, 29 May 2013; doi:10.1038/ki.2013.199.

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# Targeting of sodium–glucose cotransporters with phlorizin inhibits polycystic kidney disease progression in Han:SPRD rats

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Renal tubular epithelial cell proliferation and transepithelial cyst fluid secretion are key features in the progression of polycystic kidney disease (PKD). As the role of the apical renal sodium–glucose cotransporters in these processes is not known, we tested whether phlorizin inhibits cyst growth and delays renal disease progression in a rat model of PKD. Glycosuria was induced by subcutaneous injection of phlorizin in male heterozygous (Cy/*p*) and wild-type Han:SPRD rats. Phlorizin induced immediate and sustained glycosuria and osmotic diuresis in these rats. Cy/*p* rats treated with phlorizin for 5 weeks showed a significant increase in creatinine clearance, a lower 2-kidneys/body weight ratio, a lower renal cyst index, and reduced urinary albumin excretion as compared with vehicle-treated Cy/*p* rats. Measurement of Ki67 staining found significantly lower cell proliferation in dilated tubules and cysts of Cy/*p* rats treated with phlorizin, as well as a marked inhibition of the activated MAP kinase pathway. In contrast, the mTOR pathway remained unaltered. Phlorizin dose dependently inhibited MAP kinase in cultured tubular epithelial cells from Cy/*p* rats. Thus, long-term treatment with phlorizin significantly inhibits cystic disease progression in a rat model of PKD. Hence, induction of glycosuria and osmotic diuresis (glycuresis) by renal sodium–glucose cotransporters inhibition could have a therapeutic effect in polycystic kidney disease.

**KEYWORDS:** polycystic; phlorizin; rat; sodium–glucose cotransport

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the development of innumerable renal cysts which originate from the tubular epithelium of various nephron segments.<sup>1</sup> Cystogenesis in ADPKD reflects epithelial dedifferentiation, increased proliferation, and abnormal fluid secretion.<sup>2</sup> The compression of healthy adjacent parenchyma by the expanding cysts leads to progressive renal failure, with more than 50% of the patients reaching end-stage renal disease during their lifetime.<sup>3</sup> Insights into the pathophysiological processes that govern cyst development led to a growing number of drug candidates in ADPKD. In particular, several drugs targeting epithelial cell proliferation or the transport processes that contribute to intracystic fluid secretion have recently been proposed. These drugs include mammalian target of rapamycin (mTOR) inhibitors, somatostatin, and vasopressin type 2 receptor antagonists.<sup>4,5</sup>

The dihydrochalcone phlorizin is a natural product and dietary constituent which is found in a number of fruit trees.<sup>6</sup> For decades, it has been extensively used as a tool for physiological research. Phlorizin's principal pharmacological action is to produce renal glycosuria and—to a lesser degree—to block intestinal glucose absorption through inhibition of the Na<sup>+</sup>–glucose cotransporters (SGLTs) located apically in the proximal tubules of the kidneys and the small intestinal mucosa. The administration of phlorizin to experimental animal models results in profound inhibition of the renal SGLTs in renal proximal tubules, resulting in marked glycosuria.<sup>6</sup>

To the best of our knowledge, the role of SGLTs in the pathogenesis of polycystic kidney disease (PKD) has never been examined. We hypothesized that induction of glycosuria and osmotic diuresis by inhibiting renal SGLTs could slow cystogenesis and renal disease progression. To test this

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hypothesis, we treated heterozygous (Cy/**p**) Han:SPRD rats with phlorizin and investigated the effects of chronic glycosuria on parameters of renal function and cystogenesis. Our data demonstrate that phlorizin effectively retarded renal functional loss and cyst volume growth in this rat model of PKD.

## RESULTS

Phlorizin treatment induces glycosuria and osmotic diuresis in Han:SPRD rats

Treatment of 5-week-old male heterozygous (Cy/**p**) and wild-type (**p**/**p**) Han:SPRD rats with phlorizin (400 mg/kg/day subcutaneous in propylene glycol) induced an immediate and sustained glycosuria. The glycosuria was accompanied by a threefold increase in urine output, reflecting the increased osmolar excretion (Table 1; Figure 1a and b). In contrast, Cy/**p** and **p**/**p** rats injected with the vehicle (propylene glycol) did not develop glycosuria. Plasma glucose levels did not change in response to phlorizin (Table 1). Despite the massive polyuria, plasma osmolality and plasma sodium and chloride concentrations were stable in Cy/**p** rats at days 17 and 35 of treatment, suggesting that the animals adequately compensated for the osmotic diuresis and did not get dehydrated. Of note, phlorizin-treated Cy/**p** rats displayed a slightly enhanced urinary sodium and chloride excretion rate compared with vehicle-treated rats, as well as a 6.7% weight difference at the end of the treatment period (Table 1).

### Effect of phlorizin treatment on renal function

After 5 weeks of treatment with phlorizin, the plasma creatinine was 28% lower in male Cy/**p** rats compared with the vehicle-treated group, and the creatinine clearance was 56% higher (Table 2; Figure 1c and d). Thus, despite increased urine output and lower body weight, the decline in renal function was significantly attenuated in the phlorizin-treated rats.

Plasma blood urea nitrogen values increased to a greater extent upon treatment of Cy/**p** rats with phlorizin compared with vehicle (Table 2). However, blood urea nitrogen values also increased upon treatment with phlorizin in **p**/**p**

control rats. This is consistent with an enhanced tubular reabsorption of urea upon induction of osmotic diuresis.

As albuminuria is a marker of renal disease severity in the Han:SPRD model of PKD, we determined the amount of urinary albumin in Cy/**p** and **p**/**p** rats treated with phlorizin or vehicle by enzyme-linked immunosorbent assay (ELISA) and SDS-polyacrylamide gel electrophoresis. Figure 2 shows that albumin excretion was higher in Cy/**p** compared with wild-type **p**/**p** rats, as expected. The excretion of urinary albumin was significantly reduced upon treatment of Cy/**p** rats with phlorizin. After 5 weeks of treatment, the albumin excretion amounted to  $2.7 \pm 0.7$  mg/day in vehicle-treated Cy/**p** rats vs.  $1.0 \pm 0.4$  mg/day in phlorizin-treated animals (63.1% less in the phlorizin-treated group compared with the vehicle-treated group,  $P < 0.01$ ).

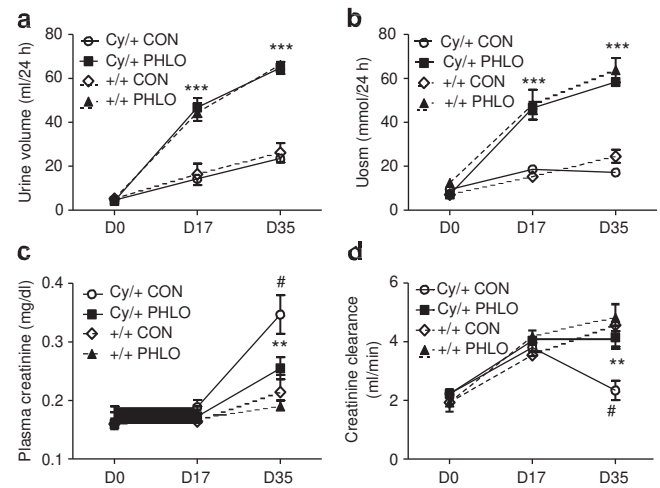


Figure 1 | Diuresis, osmolar excretion, and renal function in control (**p**/**p**) and cystic (Cy/**p**) male Han:SPRD rats treated with vehicle (propylene glycol; CON) or phlorizin (PHLO). (a) Course of diuresis, (b) 24-h osmolar excretion, (c) plasma creatinine, and (d) creatinine clearance from baseline to days 17 and 35 of treatment. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  when comparing Cy/**p** CON and Cy/**p** PHLO at each time point. # $P < 0.05$  when comparing Cy/**p** and **p**/**p** group.

Table 1 | Effect of phlorizin treatment on body weight, diuresis, and fluid and electrolyte parameters in Cy/**p** rats

	Baseline	Day 17		Day 35	
		CON	PHLO	CON	PHLO
Number of animals (n)	17	7	10	7	10
Age (in weeks)	5	7.5	7.5	10	10
Total body weight (g)	94.5 $\pm$ 2.5	212.8 $\pm$ 8.7	186.7 $\pm$ 7.3*	304.7 $\pm$ 9.4	284.2 $\pm$ 5.5*
Diuresis (ml/day)	4.5 $\pm$ 0.4	14.3 $\pm$ 1.4	47.6 $\pm$ 3.8***	23.6 $\pm$ 2.9	65.0 $\pm$ 2.2***
P <sub>osm</sub> (mosm/l)	300.5 $\pm$ 16.4	306.3 $\pm$ 5.5	306.7 $\pm$ 7.9	310.2 $\pm$ 4.1	314.2 $\pm$ 16.9
P <sub>Na<sup>+</sup></sub> (mmol/l)	143.7 $\pm$ 2.8	144.9 $\pm$ 3.6	144.4 $\pm$ 3.1	144.3 $\pm$ 3.7	144.7 $\pm$ 8.8
P <sub>Cl<sup>-</sup></sub> (mmol/l)	104.8 $\pm$ 2.3	102.6 $\pm$ 1.3	103.4 $\pm$ 2.5	104.7 $\pm$ 4.7	104.6 $\pm$ 6.8
P <sub>glucose</sub> (mmol/l)	9.3 $\pm$ 0.8	11.3 $\pm$ 1.2	11.1 $\pm$ 1.6	12.9 $\pm$ 3.0	13.0 $\pm$ 3.8
U <sub>osm</sub> (mosm/day)	8.3 $\pm$ 2.4	18.7 $\pm$ 4.0	47.0 $\pm$ 7.3***	17.2 $\pm$ 2.3	49.1 $\pm$ 13.6***
U <sub>glucose</sub> (mmol/day)	0.1	0.1 $\pm$ 0.0	21.5 $\pm$ 2.9***	0.1 $\pm$ 0.0	18.0 $\pm$ 4.6***
U <sub>Na<sup>+</sup></sub> (mmol/day)	0.6 $\pm$ 0.2	1.2 $\pm$ 0.2	1.7 $\pm$ 0.1**	1.1 $\pm$ 0.2	1.9 $\pm$ 0.8**
U <sub>Cl<sup>-</sup></sub> (mmol/day)	1.3 $\pm$ 0.4	2.7 $\pm$ 0.6	3.1 $\pm$ 0.5	2.2 $\pm$ 0.4	3.4 $\pm$ 1.1***

Abbreviations: CON, vehicle (propylene glycol) treated; PHLO, phlorizin.

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  when comparing CON and PHLO at each time point.

Table 2 | Effect of phlorizin on parameters of renal function in Cy/p and p/p rats

Group	Time point (day)	BUN (mg/dl)		Creatinine (mg/dl)		Creatinine clearance (ml/day)	
		CON	PHLO	CON	PHLO	CON	PHLO
Cy/p	Baseline	39.7±5.6	41.0±4.7	0.14±0.03	0.15±0.03	2.04±0.50	1.67±0.68
	D17	53.8±4.4 <sup>www</sup>	65.0±8.1 <sup>*ww</sup>	0.18±0.04	0.17±0.03	3.40±0.37	3.86±0.93
	D35	77.8±14.8 <sup>www</sup>	76.6±13.0 <sup>www</sup>	0.35±0.08 <sup>w</sup>	0.25±0.05 <sup>†</sup>	2.34±0.81 <sup>w</sup>	4.15±0.14 <sup>†</sup>
p/p	Baseline	38.3±3.9	35.2±4.1	0.15±0.02	0.17±0.01	1.64±0.39	1.99±0.15
	D17	38.2±4.3	50.3±12.4	0.18±0.01	0.16±0.02	3.50±0.12	4.18±0.37
	D35	36.1±2.3	53.1±10.8 <sup>*</sup>	0.22±0.03	0.21±0.03	4.56±1.26	4.82±1.01

Abbreviations: BUN, blood urea nitrogen; CON, vehicle (propylene glycol) treated; PHLO, phlorizin.

\*P<0.05 when comparing CON and PHLO at each time point. <sup>w</sup>P<0.05; <sup>ww</sup>P<0.01; <sup>www</sup>P<0.001 when comparing Cy/p and p/p at each time point for the same treatment.

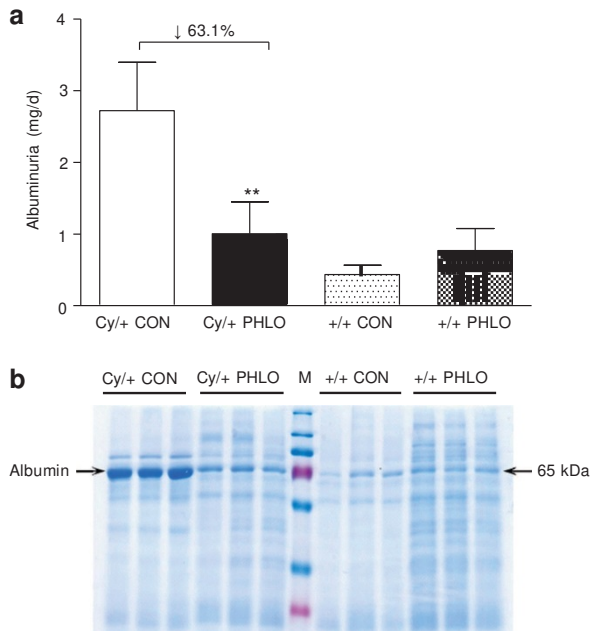


Figure 2 | Albumin excretion in Cy/p and p/p rats after 5 weeks of treatment with vehicle (propylene glycol; CON) or phlorizin (PHLO). (a) Urine albumin concentration (Cy/p CON: n¼7; Cy/p PHLO: n¼10; p/p CON: n¼5; p/p PHLO: n¼3). \*\*P<0.01; (b) Urine sample SDS-polyacrylamide gel electrophoresis. Each lane shows a urine sample of a single rat, each group shows representative samples from three rats. Loading volumes were corrected for diuresis and amounted to 15 ml for vehicle-treated rats, and 45 ml for phlorizin-treated rats. Arrow shows the band for albumin. M, marker.

This suggests that the accompanying glomerular and proximal tubular damage was strongly attenuated in phlorizin-treated Cy/p.

#### Effect of phlorizin treatment on kidney weight and morphology

After 5 weeks of treatment with phlorizin or vehicle, rats were killed and the kidneys were excised and decapsulated for weight determination and morphological analysis. The total weight of both kidneys amounted to 6.96±0.59 g in vehicle-treated Cy/p (n¼7) vs. 5.66±0.35 g in phlorizin-treated Cy/p rats (n¼10), representing a difference of 18.7% (P<0.001). Figure 3a shows that the 2-kidneys/body weight ratio was reduced by 12.6% in the phlorizin-treated rats

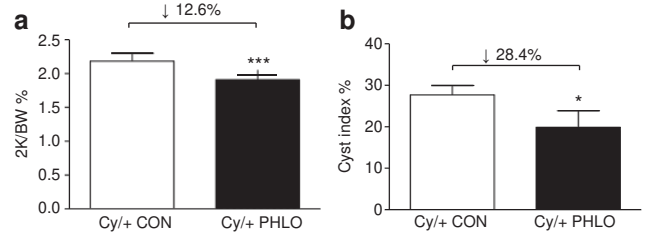


Figure 3 | Effect of phlorizin on kidney weight and cyst index. Ratio of (a) 2-kidneys/body weight (2K/BW) and (b) cyst index in male Cy/p rats treated daily for 5 weeks with vehicle (propylene glycol; CON; n¼7) or phlorizin (PHLO, n¼10). \*P<0.05, \*\*\*P<0.001.

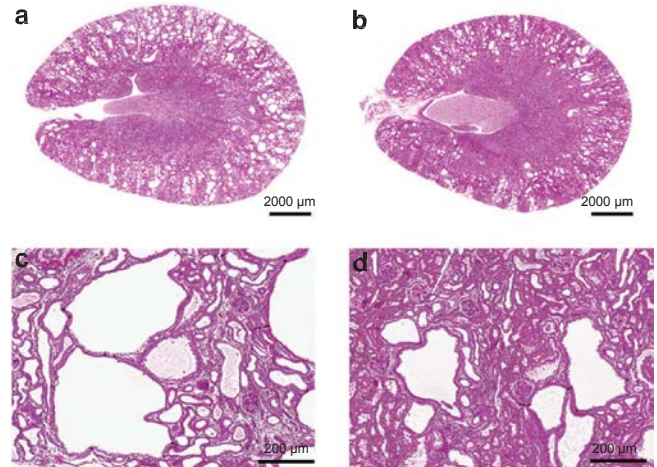


Figure 4 | Effect of phlorizin on renal histology. Renal histology (periodic acid-Schiff) in (a, c) vehicle-treated (propylene glycol; CON) and (b, d) phlorizin-treated (PHLO) male Cy/p rats. Scale bar is 2000 mm in a and b, and 200 mm in c and d.

(P<0.001). Likewise the cyst index (determined on periodic acid-Schiff-stained sections) was reduced by 28.4% in phlorizin-treated rats (P<0.05). The cyst index data were confirmed by further histomorphological analysis of the kidneys, which demonstrated that phlorizin-treated Cy/p rats had fewer and smaller cyst profiles as compared with vehicle-treated rats (Figure 4).

#### Phlorizin inhibits proliferation of cyst epithelium

Immunostaining for Ki67 was used to examine whether phlorizin treatment had an effect on cystic epithelial cell



proliferation. Figure 5 demonstrates a marked increase in the positivity of nuclei in the cystic and non-cystic epithelium of Cy/**p** kidneys in comparison with wild-type **p/p** kidneys. Quantification of the Ki67-positive nuclei revealed that the number of Ki67-positive nuclei was 39.8% lower in cystic epithelium and 23.8% lower in dilated tubules in phlorizin-versus vehicle-treated Cy/**p** rats (Table 3). These data suggest that phlorizin may directly affect cell proliferation.

Effect of phlorizin treatment on mitogen-activated protein kinase and mTOR signaling pathway  
 We then studied the effect of phlorizin treatment on the phosphorylation of two key kinases that are known to be activated in PKD, namely extracellular signal-regulated kinase (ERK; mitogen-activated protein kinase (MAPK) pathway) and S6K (mTOR pathway). Figure 6 demonstrates that the

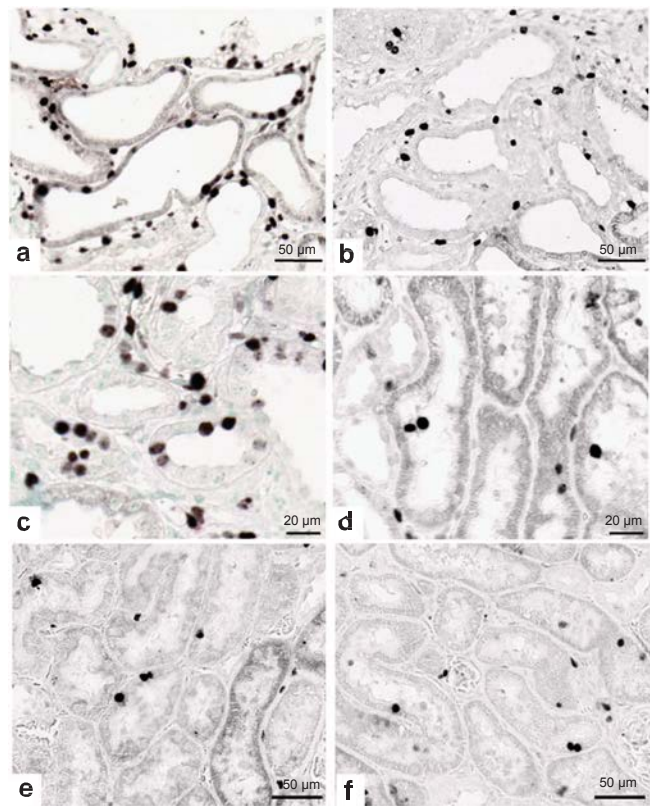


Figure 5 | Representative areas of Ki67 immunohistochemical staining in Cy/**p** rat kidneys. (a) Cy/**p** CON (cyst), (b) Cy/**p** phlorizin (PHLO; cyst), (c) Cy/**p** CON (dilated tubule), (d) Cy/**p** PHLO (dilated tubule), (e) **p/p** CON, and (f) **p/p** PHLO.

Table 3 | Effect of phlorizin treatment on Ki67 staining of Cy/**p** rat kidneys

Treatment	CON (n¼4)	PHLO (n¼7)	Difference (%)	P-value
Ki67-positive cells in dilated tubules	2.8±0.7	1.9±0.3	– 23.8	0.037
Ki67-positive nuclei in cyst-lining epithelium	5.8±2.3	3.5±1.5	– 39.8	0.029

Abbreviations: CON, vehicle (propylene glycol) treated; PHLO, phlorizin.

phosphorylation and the ratio of phosphorylated to total ERK and S6K were markedly increased in Cy/**p** compared with wild-type **p/p** rat kidneys. Of importance, phlorizin treatment was associated with a significant decrease in the phosphorylation of ERK, whereas the phosphorylation of S6K was unchanged. These data suggest that phlorizin differentially inhibits signaling cascades in the Cy/**p** kidneys.

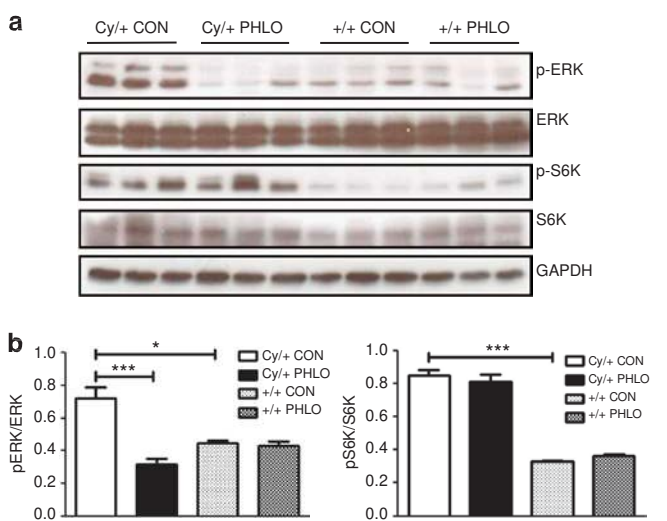


Figure 6 | Effect of phlorizin on ERK and S6K phosphorylation. (a) Western blot analysis and (b) densitometry for mammalian target of rapamycin and mitogen-activated protein kinase pathway in **p/p** and Cy/**p** kidney. Cy/**p** rats show enhanced phosphorylation of extracellular signal-regulated kinase (ERK) and S6K compared with wild-type **p/p** animals. In phlorizin-treated Cy/**p** rats, the phosphorylation of ERK and the pERK/total ERK ratio is significantly reduced, whereas the phosphorylation of S6K and the pS6K/total S6K ratio is unchanged. CON, propylene glycol treated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PHLO, phlorizin. \**P*0.05, \*\*\**P*0.001.

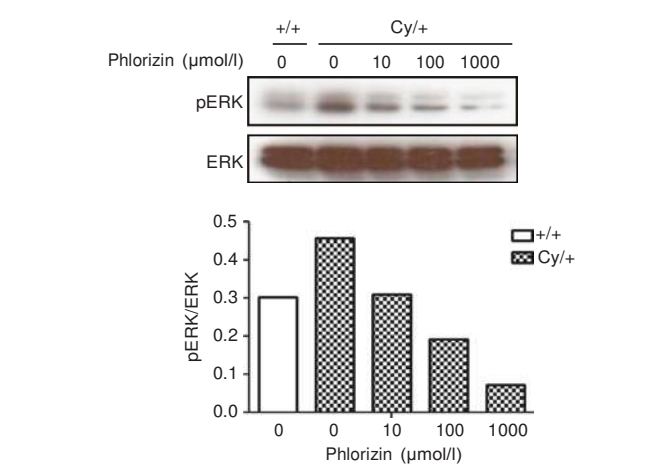


Figure 7 | Western blot analysis for phosphorylated extracellular signal-regulated kinase 1/2 (pERK; top) and total ERK (bottom) in response to phlorizin treatment (0–1000 μmol/l) in cultured Cy/**p** tubular epithelial cell (TEC). TEC cultured from **p/p** kidneys served as control. Bottom bar graph shows the densitometric pERK/total ERK ratios.

#### Effect of phlorizin on MAPK signaling pathway

To test whether phlorizin has a direct effect on MAPK activation, we examined the effect of phlorizin in cultured tubular epithelial cells (TECs) derived from Cy/**p** kidneys. Figure 7 shows that ERK phosphorylation was constitutively upregulated in TECs derived from Cy/**p** cells, contrasting with the much weaker ERK phosphorylation, which is seen in TECs that are derived from wild-type **p/p** rats. Upon incubation of the cells with phlorizin (0–1000 mmol/l), there was a dose-dependent decrease of ERK phosphorylation. This suggests that phlorizin via this direct effect on the MAPK signaling pathway could directly alter TEC activation and proliferation in PKD.

#### DISCUSSION

Treatment strategies for PKD with drugs that aim for renal-specific targets may provide superior efficacy and fewer adverse events, compared with therapeutic agents that have a broader and less selective effect. The SGLT system may be one of these renal-specific targets in PKD, and SGLT inhibitors might have a potential benefit in PKD, either by directly affecting cell proliferation or indirectly via their osmotic diuresis effect. Drugs that specifically inhibit the kidney-specific SGLT2 are currently being developed for glycemic control in type 2 diabetes.<sup>7,8</sup> Most of the SGLT2 inhibitors are structurally derived from phlorizin, which is a powerful inhibitor of both SGLT1 and SGLT2, and is known to produce massive glycosuria and osmotic diuresis in the rat.<sup>6,9</sup>

Here, we show for the first time that chronic inhibition of SGLT and induction of glycosuria with phlorizin slows renal cystic disease progression in the Han:SPRD rat model of PKD.<sup>10</sup> A 5-week treatment course with phlorizin resulted in the following: (1) improved renal function, as assessed by plasma creatinine and creatinine clearance measurements; (2) a reduction in urinary albumin excretion, which is a marker of disease progression in this model; (3) a lower total kidney weight and 2-kidneys/body weight ratio, correlating morphologically with smaller cysts and a lower cyst index; (4) decreased tubular and cystic epithelial cell proliferation, as assessed by Ki67 staining; (5) inhibition of the activated MAPK pathway, as assessed by a lower p-ERK1/2 to total ERK ratio, both *in vivo* and *in vitro*. Thus, phlorizin-mediated induction of glycosuria and the subsequent occurrence of osmotic diuresis (glycuresis) were associated with reduced cystic disease progression.

The putative mechanisms by which phlorizin inhibits cystic renal disease progression may be manifold and must be discussed at several levels. First of all, phlorizin may directly inhibit cyst epithelial cell proliferation. This is suggested by a markedly reduced number of proliferating cyst epithelial cells in phlorizin-treated Cy/**p** rats. In addition, our *in vitro* experiments with cultured Cy/**p** TECs show that phlorizin has a direct effect on the MAPK pathway, which manifests as an inhibition of ERK1/2 phosphorylation. Second, by inducing osmotic diuresis phlorizin might change the transepithelial transport of fluid and electrolytes in a way

that inhibits cyst growth. From the present studies, however, it is not possible to provide a mechanism how osmotic diuresis could reverse fluid secretion into the cysts. Third, a change in the hormonal environment (in particular the inhibition of insulin and insulin-like growth factor-1 production) is likely to be caused by the loss of glucose in the urine and/or by a direct effect of phlorizin on insulin secretion. As insulin and insulin-like growth factor-1 are known to be implicated in cyst epithelial cell proliferation, the reduced cyst development that is seen with phlorizin treatment could be partially explained by these hormonal changes.<sup>11,12</sup>

Treatment with phlorizin did not result in a stop or reversal of disease progression. The treatment was well tolerated by the rats and did not result in notable side effects, except for a threefold increase in the urine output, which was compensated by an appropriate increase in fluid intake. As the activated MAPK<sup>13</sup> and the mTOR pathway (reviewed in Torres et al.<sup>14</sup>) critically contribute to the pathogenesis of PKD, one could postulate that SGLT inhibitors might be ideal drugs to combine with drugs that affect different signaling pathways, such as the mTOR pathway that was not affected by phlorizin in this study. We and others have previously shown that treatment with sirolimus<sup>15,16</sup> or everolimus<sup>17</sup> significantly inhibited cyst growth and disease progression in Han:SPRD rats. The effect of mTOR inhibitors on cyst volume growth is only partial, perhaps because of the known compensatory upregulation of the MAPK pathway.<sup>18</sup> Combining SGLT inhibitors with mTOR inhibitors might therefore be a therapeutic strategy worthwhile pursuing in PKD.

It might be of interest to oppose the effect of phlorizin (a glycretic drug) and the effect of the vasopressin type 2 receptor antagonist tolvaptan (an aquaretic drug), the latter of which is in phase 3 clinical development for the treatment of ADPKD.<sup>19,20</sup> Both drugs target highly selective renal tubular transport systems and produce a significant and sustained increase in urine output. Similar to phlorizin, tolvaptan is also known to inhibit the ERK pathway but not the mTOR pathway.<sup>21,22</sup> At this point, it is not known whether the vasopressin-cAMP axis is altered with phlorizin. In view of the primary increase in the osmotic clearance, treatment with phlorizin could lead to increased endogenous AVP levels—an effect similar to that observed with long-term vasopressin type 2 receptor antagonism—<sup>23</sup> and this could promote distal tubular cyst growth, an effect that might not be seen in Han:SPRD as the cysts are almost exclusively of proximal tubular origin, which are not supposed to be responsive to vasopressin. Of note, increasing fluid intake and urine output experimentally by providing rats with 5% glucose in the drinking water also revealed a beneficial effect on cyst growth in a distal PKD model (PCK rat) but not in the proximal model (Han:SPRD rat).<sup>24</sup> Thus, glycretic and aquaretic drugs display similar and dissimilar effects on cyst volume progression and should be developed further as a future treatment for ADPKD.

By now there is no evidence that chronic SGLT inhibition may potentially be deleterious for the kidney. Results from *Sgt2*<sup>-/-</sup> knockout mice<sup>25</sup> and more recently from *Sgt1*<sup>-/-</sup> knockout mice<sup>26</sup> have revealed that there is no alteration in renal function and morphology. As in phlorizin-treated rats, *Sgt2*<sup>-/-</sup> mice display glycosuria, polyuria, and increased food and fluid intake without differences in plasma glucose concentrations and glomerular filtration rate compared with wild-type mice. SGLT2 deficiency is also not associated with volume depletion, as shown by similar body weight, BP, and hematocrit. Likewise, human familial renal glycosuria that results from mutations in the SGLT2 gene is considered to be a benign condition.<sup>27</sup> As far as we know, the renal safety of the SGLT2 inhibitor dapagliflozin (in clinical development for type 2 diabetes) is excellent, except for a slightly higher incidence of genitourinary infections.<sup>28</sup>

In summary, we have shown that induction of glycosuria and osmotic diuresis (glycuresis) by inhibition of the renal SGLT with phlorizin resulted in a significant retardation of the cystic disease progression in the Han:SPRD rat model of PKD. Further studies are in progress to assess the effect of glycosuria induction in additional models of PKD, including the ARPKD model in PCK rats. Whether this experimental strategy can be translated to human ADPKD remains to be determined.

## MATERIALS AND METHODS

### Animals

The Han:SPRD rat colony was established in our animal facility from a litter, which was obtained from the Rat Resource and Research Center (Columbia, MO). Heterozygous cystic (Cy/*p*) and wild-type normal (*p/p*) rats were used in this study. Only male rats were used as cysts develop more rapidly in male compared with female rats. The regulatory commission for animal studies, a local government agency, approved the study protocol. Rats had free access to tap water and standard rat diet.

### Study design

Treatment was started in 5-week-old male heterozygous Cy/*p* or wild-type *p/p* control Han:SPRD rats. Groups of 7–10 rats were used. Phlorizin (Sigma-Aldrich, Buchs, Switzerland) was dissolved in a solution of 60% propylene glycol in phosphate-buffered saline and was injected subcutaneously at a dose of 400 mg/kg/day for 5 weeks. Control rats were injected with the same volume of 60% propylene glycol in phosphate-buffered saline. The dose of the phlorizin or the vehicle was adjusted daily according to the body weight of the rats. Blood and urine were collected at baseline (before treatment), at day 17 and 35 of treatment in all animals. All samples were stored at -20 °C before measurement. Rats were killed after 5 weeks of treatment, and kidneys were harvested for further analysis.

The Synchron LX System, UniCel DxC 600/800 System, and Synchron System AQUA CAL 1 and 2 (Beckman, Brea, CA) were used for the quantitative determination of glucose, sodium, chloride, creatinine, and blood urea nitrogen concentrations in rat plasma and urine. Plasma and urine osmolalities were measured by using an Advanced freezing-point osmometer.

The GenWay (San Diego, CA) rat albumin ELISA kit was used for urine albumin concentration measurements. The urine samples

were diluted 500 times in the diluent solution. One-hundred microliter aliquots of albumin standard or urine sample were added into each well of precoated plates. Plates were incubated for 30 min at room temperature and were washed four times thereafter. Plates were then incubated with horseradish peroxidase-conjugated anti-albumin solution for 30 min at room temperature in the dark, and were washed again four times. Then 100 µl of 3,3',5,5'-tetramethylbenzidine substrate solution was added into each well, and plates were incubated in the dark for 10 min at room temperature. Then 100 µl stop solution was added, mixed well, and the absorbance at 450 nm was determined using a Tecan ELISA reader (Männedorf, Zürich, Switzerland).

### Tissue sectioning, periodic acid-Schiff staining, and cyst index determination

For the histological examination, one of the kidneys from each animal was sliced perpendicularly to the long axis at 2-mm intervals. Slices from the midportion of the kidneys were fixed in 10% buffered formalin overnight. On the next day tissues were embedded in paraffin. Three-micrometer sections were stained with periodic acid-Schiff following routine protocols. The stained sections were subjected to cyst index analysis, using the HistoQuest image analysis software (TissueGnostics, Vienna, Austria) to count the whole cortex region (total area, TA) and the cyst area (cyst area, CA) in the renal cortex. The cyst index was calculated as CA/TA×100.

### Immunohistochemistry

Immunohistochemistry for Ki67 was performed on 3-mm tissue sections. In brief, the tissue sections were deparaffinized and rehydrated. The antigen retrieval was performed in an autoclave oven. As primary antibody we used a mouse anti-Ki67 antibody (BD Pharmingen, San Jose, CA). After applying the primary antibody for 1 h the sections were washed, and then incubated with the biotinylated secondary antibody (Vector, Los Angeles, CA) for 30 min. This was followed by the application of the ABC reagent (Vector), using 3,3'-diaminobenzidine with metal enhancement as the detection reagent.

For each section we randomly chose 10 cysts, counted the number of Ki67-positive nuclei, and then averaged the number of Ki67-positive nuclei per cyst. We also chose 10 areas with dilated tubules and counted the number of Ki67-positive dilated tubules and the number of positive Ki67 nuclei per tubule, and then averaged the Ki67-positive nuclei in each positive dilated tubule.

### Western blot analysis

Kidneys were homogenized with ice-cold lysis buffer containing 40 mmol/l Hepes, 120 mmol/l NaCl, 1 mmol/l ethylenediaminetetraacetic acid, 10 mmol/l potassium pyrophosphate, 10 mmol/l glycerol phosphate, 50 mmol/l NaF, 0.5 mmol/l NaVO<sub>3</sub>, 1% Triton-X 100, and protease inhibitor mixture (pH 7.6). Tissue lysates were cleared by centrifugation. Equal amounts of lysates were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies against ERK1/2<sup>Thr202/Tyr204</sup>, ERK1/2, p70 S6K Thr<sup>421/Ser424</sup>, p70 S6K (all from Cell Signaling Technology, Danvers, MA), and glyceraldehyde-3-phosphate dehydrogenase. Sheep anti-mouse IgG-HRP and donkey anti-rabbit IgG-HRP were used as secondary antibodies. Quantification of phosphorylated ERK1/2 and p70 S6K expression was normalized for total ERK1/2 and p70 S6K, respectively, using a densitometer,



and the data are reported as ratio of phlorizin- versus vehicle-treated Cy/p and p/p rats.

### Primary cultures of TECs from Cy/p rats

Primary cultures of TECs from 8-week-old p/p and Cy/p kidneys were prepared by mincing the kidneys and digesting the tissues with 1 mg/ml collagenase with gentle agitation for 1 h at 37 °C. The suspension was allowed to sediment for 1 min. Cells were collected by harvesting the supernatant twice, and were then washed three times with 10% fetal bovine serum (FBS)/Hanks balanced salt solution. Isolated cells were resuspended in K1 medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% FBS, 10 mmol/l HEPES, 42 mmol/l sodium bicarbonate, 50 ng/ml insulin, 50 nmol/l hydrocortisone, 50 ng/ml transferrin, 5 pmol/l triiodothyronine, 20 ng/ml rat EGF, 100 IU/ml penicillin, and 100 mg/ml streptomycin). Cells were then seeded in collagen type 1-precoated culture dishes and grown to confluence. The medium was then changed to a K1 medium with 0.5% FBS for 24 h, and the cells were then incubated with phlorizin (0–1000 mmol/l) for 24 h. Lysates were then prepared and resolved by SDS–polyacrylamide gel electrophoresis and probed by western blot analysis with antibodies against ERK1/2<sup>Thr202/Tyr204</sup> and total ERK1/2 as described above.

### Statistical analysis

Data are presented as means ± s.d. Data were tested for statistically significant differences between treatment groups by using the unpaired two-tailed t-test, and by using the GraphPad Prism version 5.0 software (GraphPad, San Diego, CA).  $P < 0.05$  was considered to indicate statistically significant differences.

### DISCLOSURE

All the authors declared no competing interests.

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